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Distinction of the memory B cell response to cognate antigen versus bystander inflammatory signals

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The hypothesis that bystander inflammatory signals promote memory B cell (B_{MEM}) self-renewal and differentiation in an antigen-independent manner is critically evaluated herein. To comprehensively address this hypothesis, a detailed analysis is presented examining the response profiles of B-2 lineage $B220^+IgG^+$ B_{MEM} toward cognate protein antigen in comparison to bystander inflammatory signals. After in vivo antigen encounter, quiescent B_{MEM} clonally expand. Surprisingly, proliferating B_{MEM} do not acquire germinal center (GC) B cell markers before generating daughter B_{MEM} and differentiating into plasma cells or form structurally identifiable GCs. In striking contrast to cognate antigen, inflammatory stimuli, including Toll-like receptor agonists or bystander T cell activation, fail to induce even low levels of B_{MEM} proliferation or differentiation in vivo. Under the extreme conditions of adjuvanted protein vaccination or acute viral infection, no detectable bystander proliferation or differentiation of B_{MEM} occurred. The absence of a B_{MEM} response to nonspecific inflammatory signals clearly shows that B_{MEM} proliferation and differentiation is a process tightly controlled by the availability of cognate antigen.

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Abbreviations used: ASC, antibody-secreting cell; BCR, B cell receptor; B_{MEM}, memory B cell; GC, germinal center; IF, immunofluorescence; KLH, keyhole limpet hemocyanin; MZ, marginal zone; NF, naive follicular; NP, 4-hydroxy-3-nitrophenylacetyl; PB, plasmablast; PC, plasma cell; PE, phycoerythrin; SHM, somatically hypermutated; sPE, soluble PE; TLR, Toll-like receptor; $VACV_{WR}$, vaccinia virus Western reserve strain.

Life-long immunity is central to the survival of the host. This immunity is engendered by the persistence of memory T cells, memory B cells (B_{MEM}), and long-lived BM plasma cells (PCs; Amanna et al., 2007; Dörner and Radbruch, 2007). Long-lasting B cell–mediated immunity has been referred to as serological memory (Traggiai et al., 2003) and can be sustained by recurrent antigen exposure. In the absence of periodic exposure to antigen, it is thought that the production of inflammatory signals to unrelated antigens serve as mediators sustaining serologic memory through activation of B_{MEM} in a noncognate manner. The in vitro and in vivo response profiles of antigen-specific B_{MEM} to cognate antigen and "nonspecific" or bystander inflammatory mediators are analyzed herein to critically evaluate the mechanisms underlying the maintenance of serological memory.

B_{MEM} and long-lived PCs are the products of the germinal center (GC) reaction and express somatically hypermutated (SHM) immunoglobulin receptors of the switched isotypes (MacLennan, 1994; McHeyzer-Williams and Ahmed, 1999). After B_{MEM} exit from the GC reaction, they colonize the splenic marginal zones (MZs) and the B cell follicles, where they reside in a quiescent state for months or, likely, years (Liu et al., 1988, 1991; Schittek and Rajewsky, 1990; Anderson et al., 2007). Upon rechallenge with cognate antigen, histological studies in rats observed that B_{MEM} egress toward the T cell–rich periarteriolar lymphocytic sheath and, upon receiving T cell help, proliferate and differentiate into plasmablasts (PBs) situated in both the periarteriolar lymphocytic sheath and in the splenic red pulp (Liu et al., 1991). Kinetic studies indicate that the generation of this response is rapid and peaks ~4–5 d after secondary antigen encounter (Liu et al., 1991; McHeyzer-Williams et al., 2000; Minges Wols et al., 2007). The PB response dissipates shortly after its initiation, with a subset of PBs further differentiating into long-lived PCs resident in the BM (Liu et al., 1991; Manz et al.,

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1997; McHeyzer-Williams et al., 2006). Shortly after recall, B_{MEM} cells are once again present in their initial anatomical positions (Liu et al., 1991). Despite numerous studies carefully mapping out the events of the primary humoral immune response toward protein antigens (Jacob et al., 1991; MacLennan, 1994; Allen et al., 2007), relatively little is known about the events occurring during a secondary humoral immune response, including whether antigen reencounter results in the expression of GC markers by antigen-responding B_{MEM} and/or if B_{MEM} form GC structures en route to differentiating into PCs (Liu et al., 1991; MacLennan, 1994; McHeyzer-Williams et al., 2006). As the fate and behavior of antigen-activated B_{MEM} is critical to our understanding of B_{MEM} self-renewal, included in this study is a detailed analysis of the in vivo response of B_{MEM} toward cognate antigen.

The mechanisms mediating the survival of B_{MEM} are unknown. Although B_{MEM} require phospholipase C γ signaling through the B cell receptor (BCR) for survival, it is clear that the B_{MEM} BCR need not be permanently engaged with antigen, as B_{MEM} do not require the presence of persisting antigen or antigen complexes to survive (Maruyama et al., 2000; Anderson et al., 2006; Hikida et al., 2009). It is apparent that B_{MEM} occupy a survival niche independent from other B-2 lineage cell subsets, as, unlike all other mature cells of the B-2 lineage, they do not require BAFF and APRIL survival signals (Benson et al., 2008; Scholz et al., 2008). At the level of the organism, it is thought that humoral memory as a whole is sustained by the stem cell–like qualities of B_{MEM}, with this cell type undergoing low levels of self-renewal and replenishment of the long-lived PC pool over the lifetime of the organism (Traggiai et al., 2003). To sustain serological memory in the absence of cognate antigen, it has been hypothesized that quiescent B_{MEM} are periodically activated by Toll-like receptor (TLR) agonists or bystander T cell help to undergo self-renewal events and differentiate into antibody-secreting cells (ASCs) such as PBs and PCs in an antigen-independent manner (Bernasconi et al., 2002). A crucial element of this theory was the prediction that inflammatory stimuli associated with adaptive immune responses drive the activation of B_{MEM} in an antigen-independent (i.e., nonspecific) manner in vivo (Bernasconi et al., 2002, 2003; Traggiai et al., 2003). Whether B_{MEM} are indeed reactive to bystander inflammatory stimuli in vivo has not yet been tested from the vantage point of B_{MEM}.

In this work, we detail the ability of B_{MEM} to undergo self-renewal and to differentiate into ASCs in response to both antigen-dependent and bystander inflammatory stimuli. In studying these events, we also demonstrate that during clonal expansion in response to cognate antigen, B_{MEM} do not express a GC B cell phenotype, and that GC structures are not generated en route to differentiating into ASCs. We find that B_{MEM} are responsive to inflammatory stimuli in vitro, with activated B_{MEM} displaying a markedly enhanced capacity to differentiate into ASCs when compared with naive follicular (NF) B cells. However, in vivo, in response to TLR agonists, polyclonal T cell activation, protein vaccination, and even acute vaccinia virus infection, B_{MEM} neither clonally expand nor differentiate into ASCs, and thus appear ignorant to overt bystander inflammatory signals.

RESULTS

Antigen-specific and polyclonal B_{MEM} cells are responsive to TLR agonists in vitro and display an enhanced capacity to differentiate into ASCs when compared with NF B cells

A comprehensive analysis of B_{MEM} responsiveness to antigen and bystander inflammatory stimuli was undertaken both in vitro and in vivo. The term "bystander" is defined herein as stimuli acting on B_{MEM} independent of BCR engagement. In these studies, we tracked B_{MEM} specific for the thymus-dependent protein antigen R-phycoerythrin (PE), as immunization of BALB/c mice with this fluorophore elicits a detectable population of PE-binding B_{MEM} at a high resolution (referred to as PE-B_{MEM}; Hayakawa et al., 1987). After immunization with PE adsorbed to alum i.p., PE-B_{MEM} are readily detectable in the spleen at 0.01–0.05% of total splenocytes beginning 21 d after immunization, with this population still present 60 d after priming (Fig. S1; Hayakawa et al., 1987; Schittek and Rajewsky, 1990; Benson et al., 2008). In nonimmunized mice, a minute naive PE-binding population is observed at a 10-fold lower frequency (Fig. S1). The PE-B_{MEM} in immunized mice are predominantly $B220^+PE^+CD38^+IgD^-IgG1^+CD80^{hi}$ and express a phenotype consistent with long-lived affinity-matured B_{MEM} (Fig. S1; Anderson et al., 2007). In all experiments (unless indicated otherwise), PE-B_{MEM} from PE/alum-immunized mice were analyzed between days 60 and 90 after primary immunization.

The response of PE-B_{MEM} to inflammatory stimuli was first analyzed by examining the response profile of B_{MEM} to a panel of TLR agonists in vitro. We also included two other sources of B_{MEM} in these in vitro analyses to first ensure that the results were reproducible in multiple systems and to test whether B_{MEM} activation occurs in the absence of BCR cross-linking. The second system used B_{MEM} raised against and specific for the hapten 4-hydroxy-3-nitrophenylacetyl (NP), with this system having been previously characterized (McHeyzer-Williams et al., 1991; Lalor et al., 1992). The third system used B_{MEM} from a transgenic mouse in which the polyclonal B_{MEM} population is readily identified. This latter source allowed for the isolation of B_{MEM} without the need for BCR engagement by antigen, as when PE-B_{MEM} and NP-B_{MEM} are purified using antigen binding to denote specificity, cross-linking the BCR is inevitable.

PE-B_{MEM} cells. From PE-immunized mice, PE-B_{MEM} were first enriched by negative selection, and then electronically sorted based on a $B220^+PE^+dump^-CD38^+$ phenotype, with post-sort analysis indicating high levels of purity (Fig. S2 A).

NP-specific B_{MEM} cells. NP-B_{MEM} were generated in a transgenic mouse strain where Cre recombinase was inserted

in the Cγ1 locus, with this strain crossed with the Rosa26-EYFP reporter mouse (Casola et al., 2006). In this mouse, every post-GC B cell expresses YFP, with this strain affording the opportunity to track post-GC B cell subsets by YFP expression (Casola et al., 2006). Immunization of Cγ1-cre/Rosa26-EYFP mice with NP-keyhole limpet hemocyanin (KLH)/alum lead to the generation of NP-B_{MEM} (Fig. S2 B; Casola et al., 2006). After negative enrichment, NP-B_{MEM} were defined as $B220^+$ $NIP^+IgG1/YFP^+dump^-CD38^+$, with post-sort analysis indicating purity (Fig. S2 B).

Polyclonal B_{MEM} cells. Polyclonal B_{MEM} can be clearly enumerated in Cγ1-cre/Rosa26-EYFP mice through expression of YFP, loss of IgD and IgM, and expression of other B_{MEM} markers. NP-immunized Cγ1-cre/Rosa26-EYFP mice were negatively enriched for B_{MEM} with polyclonal B_{MEM} defined as $B220^+IgG1/YFP^+CD38^+dump^-CD80^+$ and sorted to purity (Fig. 1 C).

NF B cells. In parallel, NF B cells were defined as $B220^+CD23^+IgD^+CD38^+$ and were additionally sorted (Fig. S2 C).

The response of PE-B_{MEM}, NP-B_{MEM}, poly-B_{MEM}, and NF B cells to TLR agonists after 3 d of in vitro culture was analyzed (Fig. 1). PE-B_{MEM} and corresponding NF B cells and IL-4–only controls were stained with CFSE, whereas NP-B_{MEM} and poly-B_{MEM} with NF B cells and IL-4 only controls were stained with DDAO to track proliferation. After culture with the TLR4 agonist LPS, cells were analyzed for surface expression of B220 and CD138. The culture of all NF and B_{MEM} with IL-4 resulted in a uniform $B220^+$ population after 3 d (unpublished data). Upon culture of NF B cells and B_{MEM} with LPS, NF B cells remained primarily $B220^+CD138^-$, whereas all three sources of B_{MEM} generated a $B220^{int}CD138^+$ population of cells (~21–30% of total) representing the phenotype of a ASC (Fig. 1, A and D). The ASCs derived from the three sources of B_{MEM} were of the IgG isotype and, with the ASCs generated from PE-B_{MEM} and NP-B_{MEM}, specific for either PE or NP, respectively (Fig. 1 G and not depicted). NF B cells generated a high frequency of IgM^+ ASCs (Fig. 1 G). The overall degree of proliferation by the three sources of B_{MEM} was moderately less than NF B cells, although proliferation did occur, as indicated by comparison to the IL-4–only cultured cells (Fig. 1, B and E). The population of $B220^{int}CD138^+$ cells generated from poly-B_{MEM} arose in the daughter peaks, showing that B_{MEM} undergo cell division while differentiating into $B220^{int}CD138^+$ cells (Fig. 1 F).

The response of poly-B_{MEM} and NF B cells was analyzed to either Pam3CSK4 (TLR1/2 agonist), heat-killed *Listeria monocytogenes* (TLR2 agonist), poly(I:C) (TLR3 agonist), LPS (TLR4 agonist), flagellin from *Salmonella* typhimurium (TLR5 agonist), FSL-1 (TLR6 agonist), S-27609 (TLR7 agonist), ODN 1826 (TLR9 agonist), and IL-4. Both NF B cells and poly-B_{MEM} were responsive to agonists for TLR1/2, TLR4, TLR6, TLR7, and TLR9 (Fig. 1 I). Both poly-B_{MEM} and NF B responded poorly or did not respond to agonists for TLR2, TLR3, and TLR5 (unpublished data). For each TLR agonist that NF B cells or poly-B_{MEM} responded to, B_{MEM} generated a sizable population of $B220^{int}CD138^+$ cells, whereas NF B cells maintained a $B220^+CD138^-$ phenotype (Fig. 1 H). NF B cells also proliferated to a greater degree than B_{MEM} (Fig. 1 I). Finally, it was observed that while engagement of CD40 drove proliferation of both B_{MEM} and NF B cells, B_{MEM} did not differentiate into $B220^{int}CD138^+$ cells (Fig. 1, H–I). Collectively, these data show that B_{MEM} are sensitive to multiple TLR agonists in vitro and do not require BCR cross-linking to become activated and differentiate into ASCs. Furthermore, upon activation, B_{MEM} have an enhanced intrinsic capacity to differentiate into $CD138^+$ ASCs compared with NF B cells. Lastly, CD40 signaling appears to drive B_{MEM} proliferation, but to block differentiation in vitro.

B_{MEM} cells undergo rapid clonal expansion and differentiation into IgG^+ ASCs in response to soluble antigen

Having established that B_{MEM} are responsive to bystander stimuli in vitro, we used the PE-antigen system to compare B_{MEM} responsiveness toward cognate antigen versus bystander inflammation in vivo. We first performed a detailed analysis of the secondary humoral immune response to soluble PE (sPE) to define the behavior and kinetics of B_{MEM} activation in vivo. Unlike naive hosts, it is well-established that previously immunized mice are responsive to low-doses of antigen in the absence of adjuvant (McHeyzer-Williams et al., 2006). This heightened responsiveness results from the combined presence of antigen-reactive B_{MEM} and $CD4^+$ memory T cells. In concordance with this, we observed that injection of naive mice with 1 μg sPE (or 10 μg; not depicted) failed to elicit PE-ASCs (Fig. 2, A and B), whereas PE-immunized mice produced high numbers of IgG^+ PE-ASCs. A robust IgM ASC response was only elicited when naive or PE-immunized mice were injected with 1 μg of sPE along with the TLR9 agonist CpG ODN 1826 (CpG; Fig. 2 A). Thus, the IgG^+ PE-specific humoral response generated after sPE injection is likely the direct consequence of B_{MEM} activation. These data demonstrate that a low level of sPE given in the absence of adjuvant is capable of activating PE-B_{MEM} to differentiate into IgG^+ ASCs.

The in vivo proliferation of PE-B_{MEM} was measured by flow cytofluorimetric analysis of BrdU incorporation. PE-immunized mice were injected with sPE, and a 5-d BrdU pulse was performed starting on the day of sPE injection, followed by a chase period. BrdU incorporation by B_{MEM} was quantified at various times during and after the pulse period. After preenrichment, PE-B_{MEM} were detected by excluding CD4/CD8/IgD/IgM^+ cells via a dump gate and gating them on $B220^+PE^+$ cells, with these cells analyzed for BrdU incorporation (Fig. S3 A). PE-immunized mice receiving BrdU, but no antigen, and immunized mice receiving neither antigen nor BrdU were included as negative controls (Fig. 2 C). 2 d after sPE

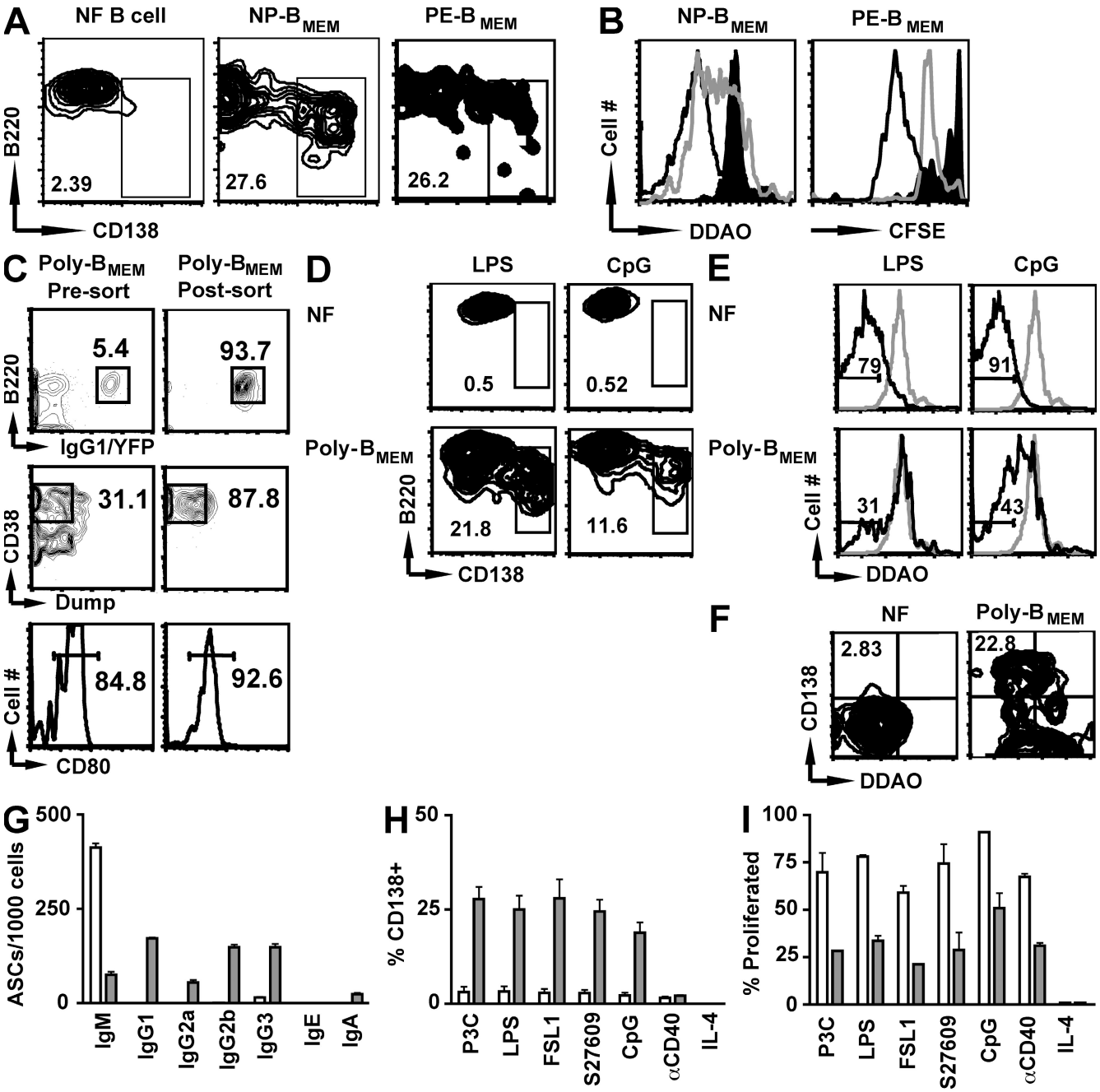

Figure 1. Antigen-specific and polyclonal B_{MEM} cells are responsive to TLR agonists in vitro and display an enhanced capacity to differentiate into ASCs compared with NF B cells. NP-B_{MEM} and PE-B_{MEM} were sorted by FACS, along with NF B cells. NP-B_{MEM} and NF B cells were stained with DDAO and PE-B_{MEM}, and NF B cells were stained with CFSE. Cells were cultured for 3 d with 10 µg/ml LPS, whereupon the cell surface phenotype was analyzed for the expression of B220 and CD138 (A) or DDAO or CFSE (B), with the percentage of $B220^+CD138^+$ cells depicted (A). (B) Filled histograms, B cells + IL-4; gray lines, B_{MEM}; black lines, NF B cells. To analyze the reactivity of B_{MEM} to TLR agonists in the absence of BCR cross-linking, poly-B_{MEM} were used. Spleens from NP-KLH–immunized Cγ1-cre/ROSA-YFP mice were enriched for B_{MEM}, with poly-B_{MEM} present in the remaining population sorted by the phenotype $B220^+IgG1/YFP^+CD38^+Dump^-CD80^+$. Dump gates, $CD43^+IgD^+IgM^+$ cells (C). Both poly-B_{MEM} and NF B cells were labeled with DDAO and cultured for 3 d in the presence of either 10 µg/ml LPS or 5 µM CpG, whereupon the cell surface phenotype was analyzed for the expression of B220 and CD138 (D) and DDAO dye dilution (E). (E) Gray lines, B cells + IL-4; black lines, NF or poly-B_{MEM} as indicated. Proliferation as a function of CD138 expression is shown, with the percentage of cells that have diluted DDAO and are expressing CD138 shown (F). The ability of cells derived from either NF B cells (open bars) or B_{MEM} (filled bars) after 3 d of LPS culture to secrete antibody of the IgM, IgG1, IgG2a, IgG2b, IgG3, IgE, and IgA isotypes was analyzed by ELISpot analysis (F). The response profiles of poly-B_{MEM} and NF B cells to a panel of TLR agonists was examined after 3 d of culture with the indicated agonist. The agonists used, their abbreviations, the receptors for which they are specific for, and the concentrations used are as follows: Pam3CSK4 (P3C), TLR1/2, 1 µg/ml; LPS (LPS), TLR4, 10 µg/ml; FSL-1 (FSL1), TLR6/2, 1 µg/ml; S-27609 (609), TLR7, 1 µg/ml; CpG ODN 1826 (CpG), TLR9, 5 µM, αCD40

rechallenge, B_{MEM} began to proliferate, with this response peaking by day 4 (Fig. 2 C and Fig. S3 A). In mice analyzed during the BrdU chase period (days 11 and 24 after sPE rechallenge), a proportion of B_{MEM} remained $BrdU^+$ at a percentage similar to that observed at the peak of the response (35.5% ± SEM 4.6 and 38.4% ± SEM 5.1 versus 48.9% ± SEM 5.8, respectively), indicating that 71–77% of B_{MEM} proliferation occurs within the first 5 d after antigen rechallenge (Fig. 2 C and Fig. S3 A). A second PE-binding population was also observed in these experiments, with this population displaying a $B220^-PE^+$ phenotype (Fig. S3 A). When the BrdU incorporation frequencies by the $B220^-PE^+$ population in PBS versus sPE-immunized mice were compared, no differences were observed between the two groups over the 24-d kinetic analysis (unpublished data). This indicates that the $B220^-PE^+$ population does not respond to sPE, and as antigen-responsiveness is a crucial element of adaptive memory, we excluded these cells from further analysis. As BrdU labeling measures the accumulation of proliferation over the time of the BrdU "pulse" period, we also analyzed B_{MEM} expression of Ki-67 at various times after antigen challenge (Fig. 2 F). Ki-67 expression is an indicator of all stages of the cell cycle and provides a snapshot of the proliferative state of the B_{MEM} at a given point in time (Gerdes et al., 1984). PE-B_{MEM} are almost uniformly $Ki\text{-}67^-$, which is indicative of their quiescent state (Fig. 2 F). By days 3 and 4 after sPE rechallenge, 76–77% of B_{MEM} cells were $Ki\text{-}67^+$. At day 5, Ki-67 expression began to dissipate, and most PE-B_{MEM} exhibited decreased Ki-67 expression on day 7, with this decrease more pronounced by day 11. By day 24, PE-B_{MEM} were once again uniform in their lack of Ki-67 expression (Fig. 2 F). In correlating the expression pattern of Ki-67 by B_{MEM} (Fig. 2 F) with the 5-d BrdU pulse followed by a chase period (Fig. 2 C), the results indicate that the majority of B_{MEM} proliferation occurs between days 2 and 5 after sPE rechallenge. In addition, the presence of $BrdU^+$ B_{MEM} 24 d after sPE injection provides direct evidence that B_{MEM} generate daughter B_{MEM} after secondary antigen encounter.

A rapid increase in the numbers of IgG^+ PE-ASCs in both the spleen and BM was observed 3 d after sPE rechallenge (Fig. 2, B and D). This burst in ASC numbers peaked 4 d after challenge in the spleen and 5 d in the BM, after which ASC numbers gradually subsided over the next 20 d (Fig. 2 D). Corresponding to the increase of ASCs after rechallenge was an increase in anti-PE IgG serum titers increasing 10-fold over the next 20 d (Fig. 2 E). These data show that ASCs are rapidly generated within 3 d after antigen rechallenge and are present to substantial degrees in both the spleen and the BM. Soon thereafter, ASC numbers declined, indicating that short-lived ASCs are present in both organs.

The observation that ~50% of B_{MEM} respond to sPE as indicated by BrdU incorporation (Fig. 2 C and Fig. S3 A) led us to question whether either the antigen dose or the absence of adjuvant during antigen administration was limiting the frequency of responding B_{MEM}. To test this, we varied the amount of sPE injected or added CpG during antigen rechallenge, with BrdU administration concurrently initiated. When analyzed 3 d later, it was observed that the dose of antigen positively correlated with the percentage of proliferating B_{MEM} (Fig. 2 G and Fig. S3 B). The addition of CpG had no further impact on the frequency of responding PE-B_{MEM} (Fig. 2 G and Fig. S3 C). However, the addition of CpG to sPE modestly increased the number of splenic IgG^+ PE-ASCs elicited after 3 d when compared with sPE alone (Fig. 2 B). In sum, these data indicate that antigen can be a limiting factor in dictating the frequency of responding B_{MEM}. Notably, TLR9 agonists failed to enhance the frequency of activated B_{MEM}. This point indicates that CpGs alone may not play a role in driving in vivo B_{MEM} activation.

B_{MEM} cells proliferate upon in vivo antigen encounter and do not express GC markers or generate GC structures

The rapid kinetics of the B_{MEM} response led us to question whether B_{MEM} form GCs after antigen encounter. Clonally expanding B_{MEM} were tracked to define their transitional stages en route to differentiating into ASCs. Under steady-state conditions, PE-B_{MEM} displayed a $GL7^-PNA^-Ki\text{-}67^-CD38^+$ phenotype as anticipated (Fig. 3 A; Lalor et al., 1992). As a positive control for GC markers, Peyer's patches were harvested and gated on $B220^+IgD^-$ cells, a population known to be primarily GC B cells, with these cells exhibiting a $GL7^+PNA^+Ki\text{-}67^{hi}CD38^-$ phenotype (Butcher et al., 1982). When PE-B_{MEM} were harvested and analyzed 3 d after sPE challenge, they were $GL7^-PNA^-Ki\text{-}67^{hi}CD38^+$, indicating these cells had responded to antigen because of Ki-67 expression, yet failed to express the GC markers GL7 and PNA (Fig. 3 A). It was plausible that the failure of antigen-responding B_{MEM} to gain a GC phenotype was caused by the solitary use of sPE, and that the induction of a GC phenotype requires the presence of an adjuvant. Therefore, CpG was added to sPE, and 3 d later, the phenotype of the responding B_{MEM} was analyzed. The addition of CpG to sPE again elicited $GL7^-PNA^-Ki\text{-}67^{hi}CD38^+$ B_{MEM} (Fig. 3 A). It was considered possible that B_{MEM} gain a GC phenotype later than 3 d after antigen encounter, so the phenotype of B_{MEM} was analyzed at later points in time. The results indicate that PE-B_{MEM} maintain a $GL7^-PNA^-CD38^+$ phenotype throughout the cell cycle (Fig. 3 B). These data define a proliferating state of B_{MEM} occurring shortly after in vivo antigen encounter,

(αCD40), CD40, 10 μg/ml; and IL-4, 10 ng/ml. The percentage of $B220^{int}CD138^+$ cells derived from NF B cells (open bars) or B_{MEM} (filled bars; H) and the percentage of cells proliferating as measured by dye dilution of DDAO (I) are depicted. Data presented is representative (A–G) or pooled (H and I) from three to five independent experiments. Purified B_{MEM} are pooled from six to eight mice per experiment, with NF B cells isolated from one mouse per experiment. Results in G–I are expressed as the mean ± SEM.

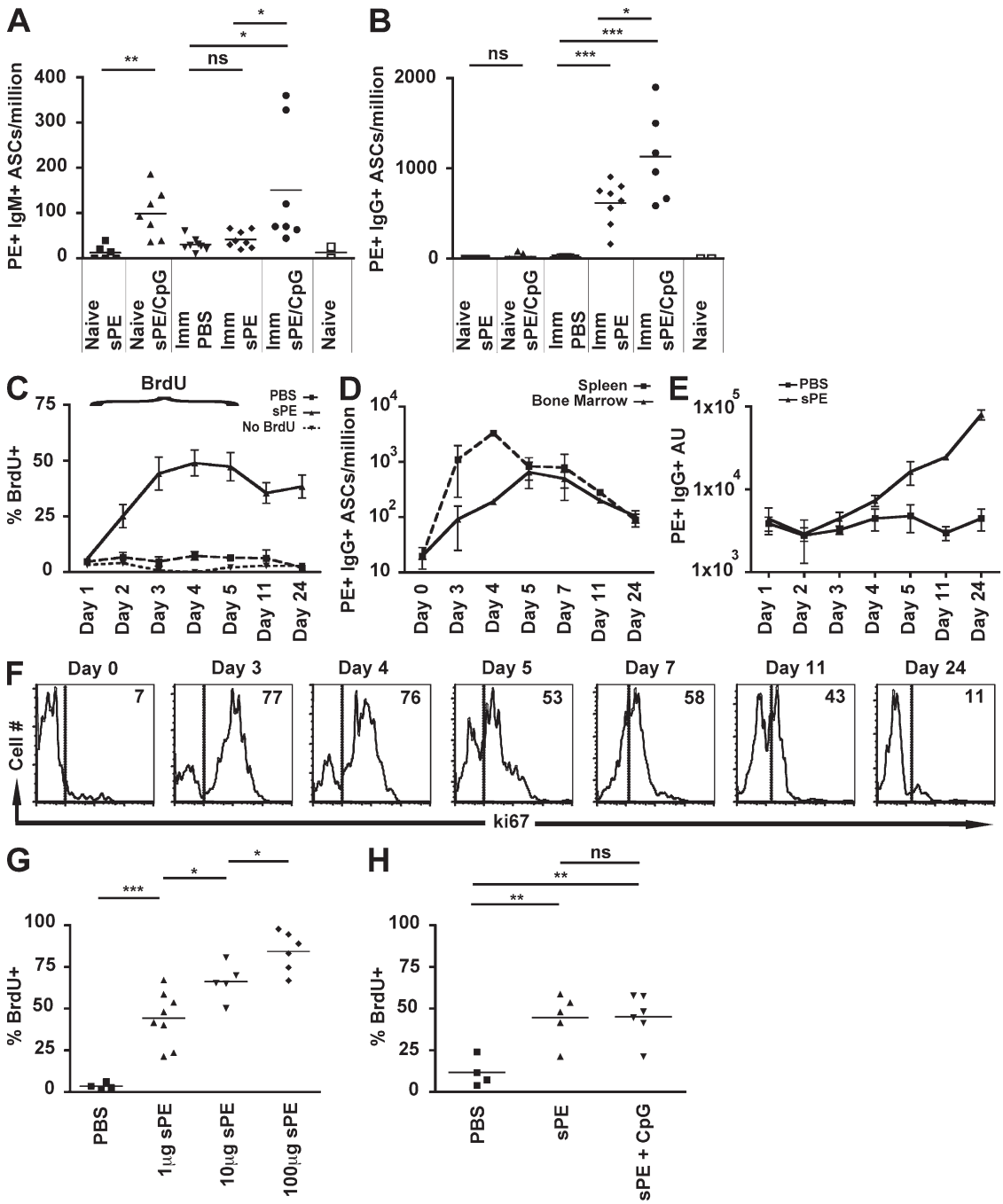

Figure 2. B_{MEM} cells undergo rapid self-renewal and differentiation into IgG$^+$ PCs in response to soluble antigen. Either naive or PE-immunized mice were challenged with PBS, 1 µg sPE, or 1 µg sPE + 50 µg CpG and 3 d later, IgM$^+$, and IgG$^+$ PE-PCs from the spleen were quantified by ELISPOT (A and B). To measure the proliferation of B_{MEM}, PE-immunized mice were injected with 5 µg sPE or PBS with BrdU added to the drinking water and continued for five days as shown (C). A group of immunized mice were included that were not given BrdU or sPE (C). At the indicated day after rechallenge, the percentage of PE-B_{MEM} incorporating BrdU was measured by FACS (C). In parallel, IgG$^+$ PE-PCs were quantified in sPE- and PBS-injected mice from both the spleen and BM by ELISpot (D). The levels of IgG$^+$ PE-specific serum antibody titers present on the indicated days after sPE injection were compared with titers present in PBS injected mice by ELISA (E). The expression levels of intracellular Ki-67 by PE-B_{MEM} (Dump$^-$B220$^+$PE$^+$; pregates not shown; Dump, CD4$^+$CD8$^+$IgD$^+$IgM$^+$) was measured at the indicated time points after sPE injection by FACS, with representative plots depicted (F). Plots were stained and analyzed on the same day. To examine the impact of varying sPE dose during rechallenge, PE-immunized mice were rechallenged with 1, 10, 100 µg sPE or PBS only, with BrdU given daily in the drinking water. After 3 d, the percentage of PE-B_{MEM} incorporating BrdU was measured by FACS (G). The same experimental setup used in G was used in H, except mice were injected with PBS, 1 µg sPE, or 1 µg PE with 50 µg CpG, with BrdU incorporation by PE-B_{MEM} again depicted. Three to five mice per experimental group were used per experiment. Data presented are representative of two to three independent experiments, with A and B pooled from two independent experiments. Error bars in C–E are expressed as the mean ± SEM. AU, arbitrary units.

with these B_{MEM} expressing a $GL7^-PNA^-Ki\text{-}67^{hi}CD38^+$ phenotype.

To investigate whether antigen-activated B_{MEM} generate GCs, GC structures were quantified in spleens where PE-B_{MEM} were either unchallenged or were responding to sPE or sPE + CpG by immunofluorescence (IF) microscopy. As a positive control for the presence of GCs, naive BALB/c were immunized with PE/alum and spleens were examined 10 d later at the height of the GC response. To ensure that PE-B_{MEM} in the sPE- and sPE + CpG–treated groups and the PE-GC B cells in the D10 PE/alum immunized groups were present and proliferating in these mice, FACS analysis was performed on half of the spleens, with BrdU incorporation by $B220^+PE^+$ cells analyzed (Fig. S4). Upon IF evaluation, in the D10 PE/alum immunized spleens, $GL7^+$ clusters were observed and proximal to $CD35^+$ follicular DCs, with these structures detected in ~50% of the B cell follicles (Fig. 3, C and D). These $GL7^+$ cell clusters were minimally present in the PBS-, sPE-, and sPE + CpG–treated groups (Fig. 3, C and D). Importantly, there was no significant increase in the number of GCs present in spleens where B_{MEM} were activated by sPE or sPE + CpG in comparison to PBS controls (Fig. 3 D). These data, coupled with the $GL7^-PNA^-Ki\text{-}67^{hi}CD38^+$ phenotype displayed by B_{MEM} after in vivo antigen-dependent activation, demonstrate that GCs are not created by B_{MEM} after antigen rechallenge.

Analysis of the in vivo B_{MEM} cell response to TLR agonists, CD40 agonists, and bystander T cell help

The in vivo B_{MEM} response to TLR agonists, CD40 agonists, and bystander T cell help was determined. PE-immunized mice were injected with PBS, sPE as a positive control, combinations of TLR agonists, agonistic αCD40, and/or αCD3. We treated mice with agonist doses at or greater than doses known to have an optimal biological effect in vivo (Ferran et al., 1990; Ahonen et al., 2004). In data where 50 μg of CpG was used, similar data were obtained using 100 μg (unpublished data). Furthermore, the efficacy of the TLR agonists was confirmed by observing an induction in the expression of CD86 by both NF B cells and $CD11c^+$ DCs 24 h after TLR agonist injection in vivo (unpublished data). PE-B_{MEM} proliferation was tracked by BrdU incorporation over a period of 3 d (Fig. S5 A). NF B cells were included in this analysis and were identified as $B220^+IgD^+$ cells (Fig. S5 B). Injection with PBS alone elicited very low amounts of BrdU incorporation by both NF B cells and PE-B_{MEM}, whereas injection with sPE elicited only B_{MEM} proliferation (Figs. 4, A and B, and Fig. S5). It was observed that both B_{MEM} and NF B cells proliferate in response to injection with agonistic αCD40 antibody alone or coupled with LPS or CpG, with the mean proliferation of both subsets as measured by BrdU incorporation between 25 and 50% (Fig. 4, A and B, Fig. S5). After injection with LPS, CpG, or αCD3 alone or αCD3 and LPS or CpG, no proliferation was observed for either NF B cells or PE-B_{MEM} (Fig. 4, A and B, and Fig. S5). These data indicate that both NF B cells and PE-B_{MEM} proliferate in response to αCD40 signals in vivo, whereas TLR agonists or activation of T cells with αCD3 did not induce B_{MEM} proliferation.

To determine whether B_{MEM} differentiate into ASCs after signaling through CD40, PE-immunized mice were injected with PBS, sPE, CpG, αCD40, or both αCD40 and CpG. IgG^+ PE-ASCs after agonist injection were quantified, and only in the sPE challenged mice was an increase in IgG^+ PE-ASC numbers observed (unpublished data). The numbers of BM IgG^+ PE-ASCs after agonist treatment were also analyzed, with serum harvested on the day of agonist injection and again when analyzed 14 d later. The only treatment group in which an increase in numbers of BM IgG^+ PE-ASCs and serum PE-specific IgG was observed in the sPE-treated cohort (Fig. 4, C and D).

These data indicate that TLR4 and TLR9 agonists and the proinflammatory cytokine storm elicited by agonistic αCD3 treatment are incapable of activating B_{MEM} and driving their proliferation or differentiation in vivo. Engagement of CD40 (either alone or coupled with TLR4 and TLR9 agonists) drives both NF B cell and B_{MEM} proliferation, but is incapable of mediating their differentiation into ASCs.

B_{MEM} cells do not proliferate or differentiate into ASCs in vivo after immunization with an irrelevant protein antigen or vaccinia virus infection

The capacity of PE-B_{MEM} to proliferate and differentiate in response to either immunization with NP-KLH/alum administered with CpG or infection with vaccinia virus Western Reserve strain ($VACV_{WR}$) was analyzed. The levels of B_{MEM} proliferation and their differentiation into ASCs were measured 17 d later with this time chosen to allow for the full development of an adaptive immune response against either NP-KLH or $VACV_{WR}$. After immunization with $VACV_{WR}$ or NP-KLH, PE-B_{MEM} did not incorporate any more BrdU (15.9% ± SEM 1.8 and 16.6% ± SEM 2.3, respectively) and then after injection with PBS alone (19.22% ± SEM 1.5; Fig. S6 C and Fig. 5 A). As anticipated, sPE induced robust PE-B_{MEM} cell proliferation, with the majority (73% ± SEM 2.8) of cells $BrdU^+$ (Fig. S6 C and Fig. 5 A). The generation of IgG^+ PE-ASCs was also measured by comparing the levels of PE^+ IgG^+ serum antibody titers present before and after immunization and by quantifying the number of IgG^+ PE-ASCs present in the spleen and BM by ELISPOT. $VACV_{WR}$ and NP-KLH immunizations did not impact the number of IgG^+ PE-ASCs present in either the spleen or the BM 17 d after immunization (Fig. 5, B and C). There was also no difference in the levels of IgG^+ PE-specific serum antibody titers after $VACV_{WR}$ or NP-KLH immunization (Fig. 5 D). Consistent

Each point in A, B, G, and H represents an individual mouse, with horizontal line representing the mean. Results of a Student's *t* test are shown as comparisons between the indicated experimental groups: ns, no significance; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

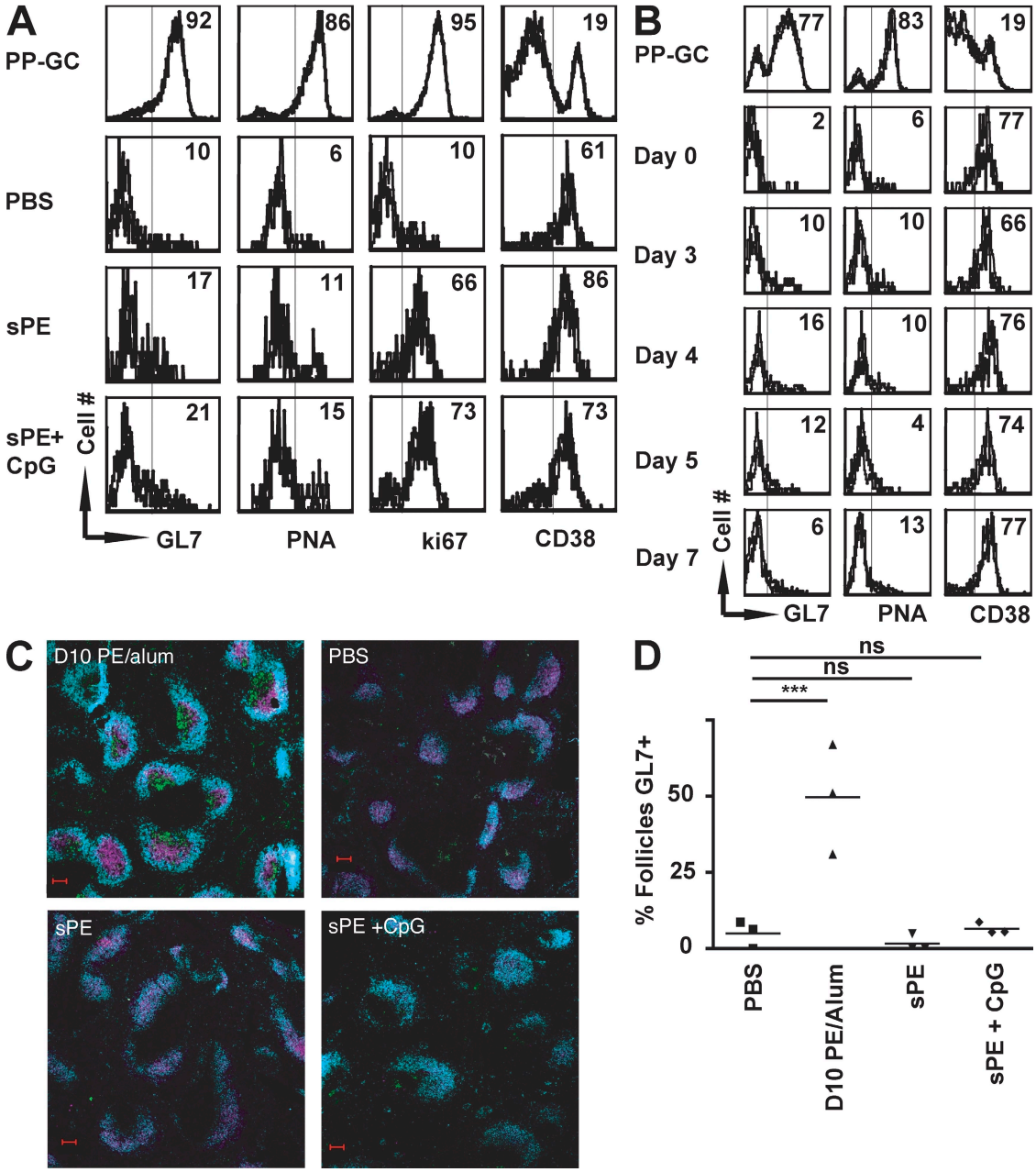

Figure 3. B_{MEM} cells proliferate upon in vivo antigen encounter and do not express GC markers or generate GC structures. PE-immunized mice were injected with PBS, 10 µg sPE, or 10 µg sPE + 50 µg CpG. 3 d later, PE-B_{MEM} ($Dump^-B220^+PE^+$; pregates not shown and Dump = $CD4^+CD8^+IgD^+IgM^+$) in each treatment group were analyzed for their expression of GL7, PNA, Ki-67, and CD38 by FACS. Included in this analysis were lymphocytes harvested from the Peyer's patches of the same mice and gated on $B220^+IgD^-$ (PP-GC; pregates not depicted). Representative data depicted are histograms of indicated phenotypic marker (A). A similar experiment was performed with responding PE-B_{MEM} examined at various time points after 10 µg sPE rechallenge (B). To detect the presence of GCs in mice harboring antigen-responding B_{MEM}, PE-immunized mice were rechallenged with PBS, sPE, or sPE + CpG. Included in the analysis were BALB/c mice that had received a primary PE/alum immunization 10 d prior. GCs in each group were quantified by IF microscopy (C) and are represented as the percentage of splenic IgD^+ B cell follicles harboring $GL7^+$ foci, with 50–75 follicles counted/spleen (D). A and B are representative of three experiments, with three to four mice analyzed on each day. The IF experiment contained three mice analyzed from two independent experiments (C and D). For IF images, green represents GL7, blue represents IgD, and purple represents CD35. Bar, 100 µm. In D, each point represents an independent mouse, with line representing the mean. Results of an unpaired Student's *t* test in D are shown as a comparison between indicated groups. ***, $P < 0.001$.

with our earlier observations (Fig. 2, D and E), sPE induced a substantial and significant increase in the number of IgG^+ PE-ASCs in both the spleen and BM (Fig. 5, B and C) and led to an ~30-fold increase in the levels of IgG^+ PE-specific serum antibody titers (Fig. 5 D). Both cellular and humoral anti-$VACV_{WR}$ immune responses were verified to have occurred by testing for the presence of IFN-γ^+ $VACV_{WR}$-specific CD4 and CD8 T cells in spleens harvested from $VACV_{WR}$-infected mice, but not naive mice, and by observing for the presence of anti-$VACV_{WR}$ IgG serum antibodies only in infected mice (Fig. S6, A and B).

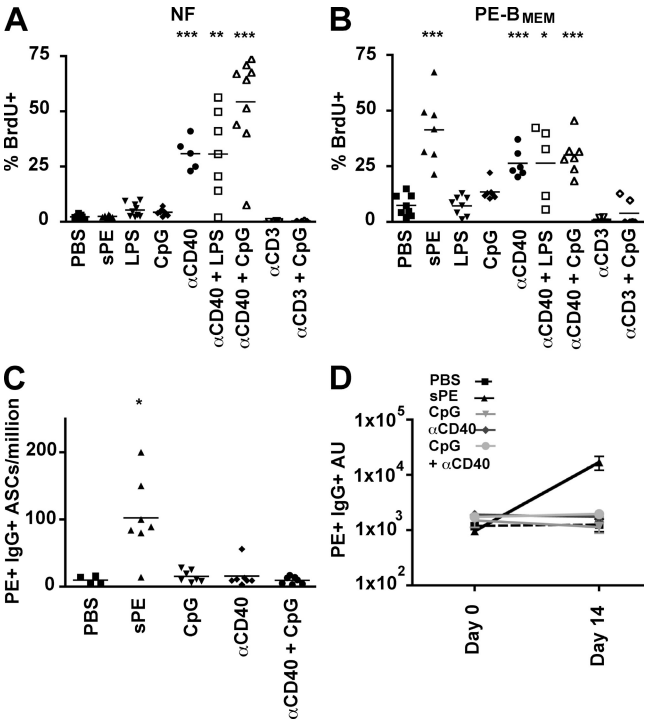

Figure 4. Analysis of the in vivo B_{MEM} cell response to TLR agonists, CD40 agonists, and bystander T cell help. PE-immunized mice were injected i.p. with PBS, 10 µg sPE, 50 µg CpG, 50 µg LPS, 100 µg αCD40, or 5 µg αCD3 either alone or in combination as indicated. For A and B, BrdU was added to the drinking water at the time of injection and for the duration of the experiment. The percentage of splenic NF B cells and PE-B_{MEM} incorporating BrdU was analyzed 3 d later by FACS (A and B, as indicated). To analyze whether CpG and αCD40 either alone or in combination enhanced the number of PE-PCs in the BM, mice received the indicated agonist and were sacrificed 14 d later, with the number of IgG^+ PE-PCs in the BM quantified by ELISpot (C). Furthermore, serum was harvested on the same day as agonist injection (Day 0), as well as on the day of sacrifice (Day 14), with the titers of IgG^+ PE-specific antibody titers analyzed on both days by ELISA (D). In A–C, three to four mice per experimental group were used per experiment. Data shown is pooled from three independent experiments. In A–C, each dot depicts an individual mouse, with mean indicated by horizontal line. In D, each group contains seven mice, with data expressed as the mean ± SEM. AU, arbitrary units. D is the pooled data of two independent experiments. Results of an unpaired Student's *t* test are shown as comparisons between the indicated experimental group and the PBS control group: no denotation, no significance. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

These data collectively demonstrate that immunization with NP-KLH and $VACV_{WR}$ infection does not induce PE-B_{MEM} proliferation or differentiation into ASCs. Thus, B_{MEM} are unresponsive to stimuli generated during a bystander immune response.

DISCUSSION

A detailed analysis of the in vivo secondary immune response of B_{MEM} in response to cognate antigen versus noncognate inflammatory bystander signals is presented. These data show that B_{MEM} respond robustly upon rechallenge with cognate antigen, but do not acquire the expression of GC markers or form GCs in response to cognate antigen. In striking contrast, the response of B_{MEM} to a battery of proinflammatory signals is remarkably silent. It has been proposed that serological

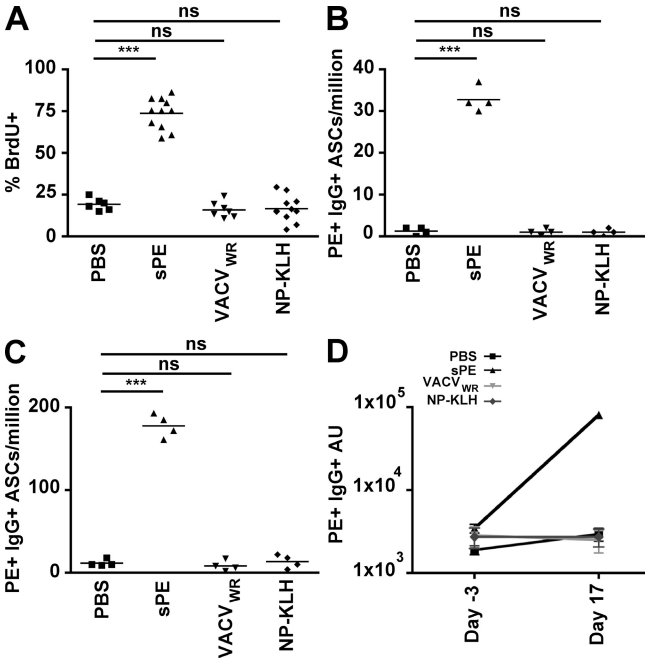

Figure 5. B_{MEM} cells do not proliferate or differentiate into PCs in vivo after immunization with an irrelevant protein antigen or vaccinia virus infection. PE-immunized mice were injected i.p. with PBS, 10 µg sPE, 2×10^6 PFU $VACV_{WR}$, or 100 µg NP-KLH/alum + 50 µg CpG, as indicated. BrdU was added to the drinking water at the time of injection and for the duration of the experiment. After 17 d, the percentage of PE-B_{MEM}–incorporating BrdU was quantified by FACS analysis (A). On the same day, the numbers of IgG^+ PE-PCs were quantified in both the spleen and BM for each treatment group by ELISpot (B and C, respectively). Serum was harvested 3 d before immunization and again on day 17, with IgG^+ PE-specific serum antibody titers from both time points shown (D). Horizontal lines represent the mean (A–C). In A–C, three to four mice per experimental group were used per experiment. Data shown in 5A is pooled from two independent experiments, and data shown in B and C is representative of one of these experiments. Results in D are expressed as the mean ± SEM. AU, arbitrary units. Seven to eight mice were used in each treatment group. D is the pooled data of two independent experiments. Results of an unpaired Student's *t* test are shown as comparisons between the indicated experimental groups in A–C. ***, $P < 0.001$.

memory is sustained by B_{MEM} responding to bystander inflammatory signals by undergoing proliferation and differentiation. However, the findings of this study indicate that B_{MEM} ignore these signals (TLR agonists, polyclonal T cell activation, aggressive vaccination, and acute viral infection), as they fail to drive B_{MEM} proliferation or differentiation in vivo.

Despite the preponderance of studies detailing the B cell dynamics of the primary humoral immune response, surprisingly few studies have detailed the events of the secondary humoral immune response. We have tracked B_{MEM} and demonstrate that cognate antigen drives the rapid clonal expansion of B_{MEM} and their terminal differentiation into ASCs, with these events occurring in the absence of GC formation. As has been widely reported, B_{MEM} rapidly differentiate to ASCs upon secondary antigen encounter (Höfer et al., 2006; Minges Wols et al., 2007), with this ASC response first detectable 3 d after antigen challenge in both the spleen and the BM and with the splenic ASC response an order of magnitude greater than that observed in the BM. This wave of ASCs dissipates after 11 d, and it is probable that the remaining ASCs in the BM 24 d after antigen challenge have been culled into the long-lived PC pool (Manz et al., 1997; Höfer et al., 2006; Minges Wols et al., 2007). These data detail the activation of B_{MEM} and define their proliferation as occurring between days 2 and 5 after antigen encounter. Proliferating B_{MEM} occupy a transitional state defined as antigen-binding $B220^+CD38^+Ki\text{-}67^+GL7^-PNA^-$ before generating quiescent daughter B_{MEM} and/or differentiating into ASCs. This blasting state is unique because of the presence of high levels of CD38 expression by proliferating cells; CD38 is a phenotypic marker previously used to denote quiescent NF B cells and B_{MEM} and is lost upon B cell activation and entry into the GC (Oliver et al., 1997). Our finding that B_{MEM} cells do not form or enter a GC reaction during their differentiation and expansion is consistent with classical studies indicating that B_{MEM} might not undergo further SHM and isotype switching upon rechallenge with antigen (Askonas et al., 1970; Okumura et al., 1976; Siekevitz et al., 1987). In these studies, limiting numbers of hapten-specific "parental" B_{MEM} were adoptively transferred together with carrier-primed T cells into lightly irradiated hosts, followed by immunization of the hosts with soluble hapten-carrier antigen. Upon analyzing the isotype distribution and SHM exhibited by the parental B_{MEM} and their progeny generated in response to cognate antigen, no change in diversity in either measure was observed. Our data showing that B_{MEM} neither gain GC markers nor seed GCs are in concordance with these studies. It is apparent that B_{MEM}, at least in these studies and in our presented data, are not instructed to enhance the affinity or isotype distribution of their antigen receptors during secondary antigen encounter.

Our data indicate that quiescent IgG^+ B_{MEM} are not activated by TLR agonists or bystander T cell help, and thus do not undergo self-renewal events or differentiate into ASCs in an antigen-independent manner. We should note that our analysis was limited to IgG^+ PE-B_{MEM} and that our experimental system excluded testing whether IgM^+ B_{MEM} are responsive to bystander inflammatory signals. There is a growing body of evidence both in favor of and in conflict with the hypothesis that serological memory is sustained by the activation of B_{MEM} by bystander inflammation. Evidence in favor of this theory includes in vitro observations in which human B_{MEM} were shown to have increased sensitivity and to selectively proliferate and differentiate into ASCs in response to bystander stimuli as compared with NF B cells (Bernasconi et al., 2002, 2003). In vivo studies in humans found that for single-pathogen specificities, serum antibody levels linearly correlated with the frequency of B_{MEM} present in a host, indicating that the ASC compartment was genealogically linked to and undergoing replenishment by the B_{MEM} compartment (Bernasconi et al., 2002). Mouse studies also supported this link between the B_{MEM} and ASC compartments as elimination of virus-specific B_{MEM} led to a slow decay of virus-specific ASC numbers over time in comparison to mice still containing B_{MEM} (Slifka et al., 1998). Lastly, it was observed that during an immune response against a single pathogen, an increase in both blood ASC numbers and serum antibody levels occurred for specificities to other pathogens (Bernasconi et al., 2002). This result was interpreted as evidence that host B_{MEM} were recipients of antigen-independent stimuli and responded by differentiating into ASCs.

Despite these data suggesting that B_{MEM} respond to bystander inflammation, more recent studies have put this hypothesis under scrutiny. In particular, further attempts to correlate antigen-specific B_{MEM} numbers with circulating ASCs in human subjects have been unsuccessful (Amanna et al., 2007). Mouse studies where long-lived ASC numbers were quantified after B_{MEM} depletion observed no decay in ASC numbers over time, contrary to initial reports (Ahuja et al., 2008; DiLillo et al., 2008). Lastly, a series of studies reported that in mice that were administered a TLR agonist or in humans that were given a vaccine, ASC numbers and serum antibody levels against specificities other than the immunizing antigen did not increase (Di Genova et al., 2006; Amanna et al., 2007; Xiang et al., 2007; Richard et al., 2008).

Our studies show that the in vitro sensitivities of B_{MEM} to activation are not representative of their sensitivities in vivo and cast doubt on the hypothesis that B_{MEM} can be activated by bystander inflammation. Our in vitro studies examining the response profiles of murine B_{MEM} to both antigen-dependent and -independent stimuli extended prior studies using human (Arpin et al., 1997; Bernasconi et al., 2002) and murine (Richard et al., 2008) B_{MEM}, as we observe that murine B_{MEM} are sensitive to TLR agonists and have an enhanced intrinsic ability to differentiate into ASCs upon activation with TLR agonists, regardless of whether the BCR is engaged by antigen. Given these data, and our knowledge of the robust response of B_{MEM} to cognate antigen in vivo, an exploration of the response of B_{MEM} to several nonspecific inflammatory signals in vivo demonstrated that B_{MEM} are in fact unresponsive to these stimuli. The first panel of signals tested was derived from activated T cells and included CD154

expression by these cells, as well as proinflammatory cytokines either alone or together with TLR agonists. We first analyzed the impact of agonistic αCD40 antibodies on B_{MEM} proliferation and differentiation. The injection of mice with αCD40 alone or with TLR4 or TLR9 agonists elicited substantial levels of B_{MEM} and NF B cell proliferation. Despite this proliferation, these B_{MEM} did not differentiate into ASCs. These data are in agreement with our in vitro observations, where αCD40 induced the proliferation of, yet failed to differentiate, poly-B_{MEM} into ASCs. These studies also extend previous in vitro studies where CD40 signaling triggered human B_{MEM} proliferation, but impeded their differentiation into ASCs (Arpin et al., 1995). Using a second approach, we activated T cells in vivo through the injection of agonistic αCD3 antibodies, with this treatment given either alone or with TLR agonists. Injection of this antibody is known to elicit rapid and transient activation of T cells, which release a proinflammatory cytokine storm that includes IL-2, IFN-γ, and TNF (Ferran et al., 1990; Scott et al., 1990). These stimuli did not induce either B_{MEM} proliferation or differentiation.

It is highly likely that the in vivo response of B_{MEM} to overt antibody-mediated CD40 signaling does not represent the hypothetical situation where B_{MEM} and activated T cells transiently engage in a CD40/CD40L synapse independent of an antigen bridge. As we found no B_{MEM} proliferation upon vaccination with an irrelevant thymus-dependent antigen or infection with vaccinia virus (a situation generating large numbers of activated CD4 T cells expression CD40L), it is probable that this interaction does not occur and is thus not a mechanism for propagating B_{MEM} renewal. Instead, this data should be interpreted as in vivo proof that CD40 signaling drives B_{MEM} proliferation but blocks their differentiation into ASCs, indicating that differentiation occurs upon cessation of CD40 signaling.

Immunization with NP-KLH, along with alum and CpG, and infection with vaccinia virus results in the acute induction of both innate and adaptive immunity. B_{MEM} ignored the stimuli associated with these responses and neither proliferated nor differentiated into ASCs during the multi-week period in which these immune responses were occurring. In summary, inflammatory signals associated with TLR signaling, T cell activation, and adaptive immune responses are incapable of driving B_{MEM} proliferation or differentiation into ASCs.

The possibility remains that B_{MEM} and long-lived PCs might survive for the lifetime of the host, with these populations not requiring self-renewal or replenishment and with serological memory not requiring maintenance. However, this hypothesis requires that B_{MEM} and PCs survive for perpetuity, and this quality appears unrealistic. From our presented work, we show that B_{MEM} do not clonally expand or differentiate in response to bystander inflammatory signals. It is possible that there are other signals capable of inducing in vivo B_{MEM} expansion and differentiation that are not included in this analysis. Alternatively, the maintenance of serological memory may occur via a stochastic rather than an induced process, with B_{MEM} not requiring an extrinsic signal to maintain serological memory such as those tested herein.

MATERIALS AND METHODS

Mice and immunizations. These studies were approved by the Institutional Animal Care and Use Committee of Dartmouth College (Lebanon, NH). BALB/c mice were purchased from the National Cancer Institute. $RAG^{-/-}$ mice were purchased from The Jackson Laboratory. Cγ1-cre mice crossed with Rosa26-EYFP mice have been previously described and were provided by K. Rajewsky (Harvard Medical School, Boston MA) and S. Casola (Fondazione Italiana per la Ricerca sul Cancro Institute of Molecular Oncology Foundation, Milan, Italy; Casola et al., 2006). All animals were maintained in a pathogen-free facility at Dartmouth Medical School. For primary immunizations, 10 μg of R-PE (Cyanotech) or 100 μg of NP_{28}-KLH (Biosearch Technologies) adsorbed to prepared alum was injected i.p. in a volume of 200 μl. For secondary challenge, 10 μg of PE (unless indicated otherwise) in PBS in a volume of 200 μl was injected i.p. For $VACV_{WR}$ infections, virus was propagated and titered on the 143B cell line as previously described (Fuse et al., 2008), with 2×10^6 PFU injected i.p. For BrdU treatment, 0.8 mg/ml was added to the drinking water and changed daily except in Fig. 5 where BrdU was changed every other day.

Cell preparation. Single-cell suspensions of lymphocytes were prepared as previously described (Benson et al., 2008). For enrichment of splenocytes for B_{MEM} before phenotyping and BrdU analysis, $CD4^+$, $CD8^+$, IgD^+, and IgM^+ cells were removed by negative depletion using a mix of biotinylated antibodies. Cells were removed using the EasySep Biotin Selection Kit for Mouse Cells (StemCell Technologies). For enrichment of B_{MEM} before cell sorting, IgD^+ and IgM^+ cells were first removed, and then $CD43^+$ cells were removed in a two-step process using the EasySep Mouse B Cell Enrichment Kit.

Cell culture/reagents. B cells were cultured in RPMI media supplemented with 10% FBS (Atlanta Biologicals), Hepes, 50 μM β-ME, and penicillin/streptomycin/L-Glutamine. Sorted B cells were cultured in 96-well round-bottomed plates with 10,000–15,000 cells/well and were cultured at 37°C. For in vitro $VACV_{WR}$ CD4 and CD8 recall, BALB/c splenocytes were infected for 2 h with a 10:1 ratio of virus to cells. Infected cells were then plated in a round-bottom 96-well plate with 5×10^6 splenocytes harvested from either D17 $VACV_{WR}$-infected or noninfected mice added and cultured for 6 h in the presence of 10 μg brefeldin-A and 10 U/ml IL-2. Intracellular staining of IFN-γ was performed as previously described (Fuse et al., 2008). The following reagents and vendors were used in this study: CpG-ODN1826 (5′-TCCATGACGTTCCTGACGTT-3′) with phosphorothioate bases (Eurogentec S.A.), LPS 055:B5, BrdU (Sigma-Aldrich), Pam4CSK4, HKLM, poly(I:C), ST-FLA, and FSL1 (Invivogen), S-27609 (3M Pharmaceuticals), IL-4 (Peprotech), αCD40 clone FGK45, and αCD3 clone 145 2C11 antibodies (Klaus Lube; BioExpress).

Flow cytometry. Antibodies against the following antigens were used: B220 (clone 6B2), IgG1 (clone A85-1), IgD clone (11-26c), IgM (clone 11–41), CD4 (clone GK1.5), CD8 (clone 2.43), IFN-γ (clone XMG1.2), CD43 (clone 1B11), CD38 (clone 90), CD23 (clone B3B4), CD80 (clone 1610-A1), Ki-67 (clone B56), CD138 (clone 281–2), BrdU (clone PRB-1), CD35 (clone 8C12), GL7, and PNA. NIP-PE and PE-binding cells were detected by staining with NIP_{20}-PE (generated in-house) or with 2 μg/ml PE. For CFSE or DDAO dye dilution, cells were labeled with 5 μM CFSE or 10 μM DDAO in RPMI at 37°C for 10 min (Invitrogen). Intracellular BrdU (BD) and Ki-67 (eBioscience) staining was performed according to the manufacturers recommendations. Flow cytometry was performed on a refurbished FACSCAN running CellQuest software (BD), with data analysis performed using FlowJo (Tree Star, Inc.). Cell sorts were performed on a FACSAria (BD; Flow Cytometry Facility at Dartmouth Medical School).

ELISpot/ELISA analysis. For ELISpot analysis, cells were apportioned to PE, NP_3-BSA, or polyclonal anti-Ig–coated Multiscreen 96-well plates (Millipore) with twofold serial dilutions made before incubation for 7–18 h at 37°C. PCs were detected by HRP-conjugated or biotinylated anti–mouse IgM, IgG1, IgG2a, IgG2b, IgG3, IgE, or IgA polyclonal antibodies (SouthernBiotech.), with streptavidin-HRP used in a second step when necessary. ELISpots were developed as previously described (Benson et al., 2008).

For ELISA analysis, plates were coated with either 10 μg/ml PE or 4×10^6 PFU of H_2O_2-inactivated $VACV_{WR}$ (Hammarlund et al., 2008) overnight in PBS, blocked with PBS + 5% FBS, and washed, and then 1:2,000 diluted serum added at serial 1:2 dilutions. Serum from either a PE hyperimmunized mouse or from a $VACV_{WR}$ mouse was included on each plate as a reference between plates and between experiments and used to generate a standard curve, with these serum allotted the value 8,000 arbitrary units. Antibody levels were detected with IgG-AP (SouthernBiotech) and developed with 1 mg/ml pNPP (Sigma-Aldrich) in 0.05 sodium carbonate buffer.

Immunofluoresence microscopy. Spleens were presoaked in 15% sucrose/PBS for 30 min and snap-frozen in OCT compound (Sakura Finetek) in a dry ice/ethanol slurry and stored at −80°C. Tissues were sectioned onto slides using a cryostat with 8-μm-thick sections. After drying at room temperature, the tissue was fixed in ice-cold 1:1 acetone/methanol mix for 10 min and allowed to dry, after which sections were outlined with an ImmEdge Pen (Vector Laboratories). Sections were blocked with 10% heat-inactivated normal rat serum/PBS for 1 h, washed, and stained with IgD-Alexa Fluor 647, GL7-FITC, and CD35-biotin, followed by staining with SA-PerCP. After washing, slides were mounted with PermaFluor Aqueous Mounting Media (Thermo Fisher Scientific). Samples were analyzed on a LSM 510 META confocal microscope using LSM software (Carl Zeiss, Inc.).

Online supplemental material. Fig. S1 shows FACS gating scheme and phenotype of PE-B_{MEM}. Fig. S2 displays the FACS profiles and gating schemes used to sort-purify PE-B_{MEM}, NP-B_{MEM}, and NF B cells. Fig. S3 contains FACS profiles showing BrdU incorporation by PE-B_{MEM} and data corresponding to experiments presented in Fig. 2. Fig. S4 contains FACS plots showing BrdU incorporation by PE-B_{MEM} and D10 GC B cells, with these data corresponding to experiments presented in Fig. 3. Fig. S5 contains FACS plots showing BrdU incorporation by PE-B_{MEM} and NF B cells after in vivo administration of TLR agonists, CD40 agonists, and bystander T cell help. Data correspond to experiments presented in Fig. 4. Fig. S6 depicts the presence of $VACV_{WR}$-specific CD4 and CD8 cells and $VACV_{WR}$–specific serum IgG in infected hosts. FACS plots are also depicted showing levels of BrdU incorporation by PE-B_{MEM} after protein vaccination or viral infection, with data corresponding to experiments depicted in Fig. 5. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090667/DC1.

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